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Inventor(s)	:	Donald Bissett
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Examiner	:	Roy P. Issac
Docket No.	:	9176R
Confirmation No.	:	2224
Customer No.	:	27752
Title	:	Regulation of Mammalian Keratinous Tissue Using Hexamidine Compositions

DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Washington, D.C. 20231

Dear Sir:

I, Rosemarie Osborne, do hereby declare as follows:

1. I am a graduate of Harvard University having received a Ph.D. in Pharmacology from said institution in 1983. I have been employed by The Procter & Gamble Company, assignee of the present application, since October 1, 1987. In this employment, I have led development of non-animal *in vitro* methods to evaluate skin and cornea responses to chemicals and products, utilizing human skin and cornea cell culture models. Models and methods I have developed are now international industry and regulatory standards for *in vitro* testing for safety and efficacy endpoints. I have served on government and industry expert panels on *in vitro* testing methods in the United States and Europe, and have given presentations on the topic of non-animal methods for skin and cornea safety at government agencies including the National Institutes of Health, the Environmental Protection Agency, and the Food & Drug Administration. I have published over 30 articles and book chapters, and have given

numerous presentations at international scientific meetings on the topics of skin and eye safety, *in vitro* models for skin and cornea testing, and *in vitro* methods for skin anti-aging.

2. I am familiar with the subject matter and the claims of the present application.

3. Background

This declaration describes the results of an experiment to evaluate skin effects of two compounds, hexamidine isethionate, a hexamidine salt referred to in this document as Hexamidine, and Niacinamide, a vitamin B_3 compound. The test system used was a tissue engineered *in vitro* model of human skin. To produce this skin model, living human skin cells are grown *in vitro* so that they produce layers of natural skin, including differentiated layers of the keratinous epidermis and its outermost stratum corneum barrier layer. The stratum corneum surface of these cultures can be treated with test chemicals and products to simulate topical exposures *in vivo*. These types of skin cultures are accepted as surrogates for natural human skin for transplantation in burn and skin ulcer patients[1,2,3,4] and for *in vitro* testing to assess the effects of chemicals and products[5,6,7,8], including tests that are international government regulatory agency standards[9].

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[1] Bello YM, Falabella AF, Eaglstein WH. Tissue-engineered skin. Current status in wound healing. *Am J Clin Dermatol* 2001 2(5):305-13.

[2] Horch RE, Kopp J, Kneser U, Beier J, Bach AD. Tissue engineering of cultured skin substitutes. *J Cell Mol Med* 2005 Jul-Sep;9(3):592-608.

[3] Shakespeare PG. The role of skin substitutes in the treatment of burn injuries. *Clin Dermatol.* 2005 Jul-Aug 23(4):413-8.

[4] Supp DM, Boyce ST. Engineered skin substitutes: practices and potentials. *Clin Dermatol.* 2005 Jul-Aug 23(4):403-12.

[5] Osborne R, Perkins MA. An approach for development of alternative test methods based on mechanisms of skin irritation. *Food Chem Toxicol* 1994 Feb 32(2):133-42.

[6] Fentem JH, Botham PA. ECVAM's activities in validating alternative tests for skin corrosion and irritation. *Altern Lab Anim* 2002 Dec 30 Suppl 2:61-7.

[7] Ponec M. Skin constructs for replacement of skin tissues for in vitro testing. *Adv Drug Deliv Rev* 2002 Nov 1 54 Suppl 1:S19-30.

[8] Weiss T, Basketter DA, Schroder KR. In vitro skin irritation: facts and future. State of the art review of mechanisms and models. *Toxicol In Vitro* 2004 Jun 18(3):231-43.

[9] OECD, 2004. OECD guideline for testing of chemicals, No. 431: In Vitro Skin Corrosion: Human Skin Model Test. Organisation for Economic Co-operation and Development, Paris, France.

In the experiment described in this declaration, gene expression was evaluated using a microarray approach. These microarrays are based on learnings from the Human Genome Project, which provided the sequence of the entire human genome, *i.e.*, the DNA genetic code material in human cells. From this Project, it was learned that there are over 20,000 genes contained in the human genome. The DNA in these genes serves as the template for synthesis of messenger RNA (mRNA) in the nucleus of cells, in a process called transcription. The mRNA is further modified to produce its mature form, in a process called RNA splicing. The mature mRNA is used as a template in another compartment of the cells, the cytoplasm, to produce proteins in a process called translation.

Evaluation of the types and amounts of mRNAs in cells gives an indication of which genes are being expressed, and to what extent. Human microarrays contain detectors, called probe sets, to measure the mRNAs in human cells, such as human skin cells. The particular microarrays used in the experiment described in this declaration contained probe sets that can detect the entire complement of genes contained in the human genome. Detection of mRNAs as biological indicators, or biomarkers, of how cells such as skin cells respond to a treatment is an accepted way of defining how an overall human organ such as skin responds to a treatment. This type of biomarker analysis is consistent with the use of diagnostic biomarkers to detect human cell responses as recommended by the Biomarkers Definition Working Group of the National Institutes of Health[10].

The processes of gene expression to produce mRNA, RNA splicing, and protein production are fundamental to responses of cells such as skin cells to treatments that improve the condition and appearance of keratinous tissues such as skin. The measurement of mRNAs as biomarkers using a microarray approach provides

[10] NIH Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clinical Pharmacol Therapeutics* 2001 Mar 69(3):89-95.

understanding of biological processes and pathways affected by test chemicals or products, that lead to overt changes such as the skin's condition and appearance.

4. General Overview of the Experiment

Described herein are results from an experiment to evaluate the effects of Hexamidine (H) and Niacinamide (N), alone and in combination, in human skin equivalent cultures. Gene expression profiling, that is measuring the levels of expression of mRNAs from genes, was conducted on treated cultures using Affymetrix GeneChip microarrays. More than 300 genes in the epidermis were significantly synergistically regulated by the Hexamidine + Niacinamide combination. Unexpectedly, there was a synergistic up-regulation of genes involved in gluconeogenesis (generation of the energy compound glucose) and RNA splicing, and a down-regulation of a large number of genes involved in protein degradation or catabolism. These synergistic effects may result in an enhancement of energy levels in the skin and of the synthesis and stabilization of proteins involved in cellular metabolism and skin cell structure in the epidermis. These are fundamental processes that are required to regulate the biological condition of keratinous tissues such as skin, that would include providing the skin structural protein changes essential toward preventing, retarding, and/or treating the appearance of fine lines and/or wrinkles, sagging, skin atrophy, skin dryness, regulating and/or reducing the size of pores, and/or desquamation, exfoliation and skin turnover. In addition, processes to increase cellular energy are also directed toward preventing, retarding and or treating overt changes such as fine lines and/or wrinkles; hyperpigmentation such as post-inflammatory hyperpigmentation; sagging; skin atrophy; skin dryness; dark under-eye circles and puffy eyes; sallowness; desquamating, exfoliating, and/or increasing skin turnover; enlarged pores; and/or oily and/or shiny appearance of skin.

5. Details of Work Performed

An experiment was conducted to evaluate the effects of Hexamidine and Niacinamide on skin cells at the level of gene expression. The test system was MatTek (Ashland,

MA) full thickness human skin equivalent cultures. The *in vitro* skin cultures (n = 4 for each treatment) were treated topically for 24 hours with 100 ul of solutions containing the water vehicle control (VC), hexamidine isethionate [a hexamidine salt referred to as hexamidine (H) in this document, 0.1%] along with its solvent pentanediol in a water solution, niacinamide (5%, N, a vitamin B_3 compound) in a water solution, or the combination of H + N (HN) in a water solution. At the end of the treatment period, the epidermal layer was peeled from the dermal layer with fine forceps, to obtain a purified epidermal cell preparation.

The epidermal mRNA was isolated and analyzed using microarrays (Affymetrix, Santa Clara, CA). The HG-U133 Plus 2.0 GeneChip microarray used has 54613 probe sets, which detect the transcripts (*i.e.*, mRNAs) of about 20,000 known genes as well as a large number of expressed sequences not yet associated with a known biological function. The mRNA was isolated from the skin equivalent cultures using a standard Trizol method, and was labeled and analyzed with the GeneChip microarrays according the manufacturer's (Affymetrix) protocols.

The analysis of the gene expression data sets involved a standard series of steps starting with a quality assurance process; statistical analysis of the genes' expression signals to identify which genes are expressed and significantly regulated by treatment; and identification of patterns in the gene expression data (called 'gene expression profiling') and biological interpretation. Gene expression profiling focuses on genes that are differentially expressed in treated samples relative to a control. Typically, the ratio of the value from a treated group is divided by the control value, to calculate the fold-change in gene expression induced by the treatment, *e.g.*, 2-fold indicates the level of a gene's expression in a treated culture is twice the level in a control culture. The log of the fold change is useful for analysis and visualization because the absolute values are symmetrical in the cases of up- or down-regulation relative to the control. An analysis of variance (ANOVA) was the statistical model used to identify

statistically significant interactions between H and N, with significance set at the standard $p < 0.05$.

The data set was filtered to identify which, if any, genes demonstrated a synergistic expression in response to the combination of H + N, and if so, what biological processes were affected. Synergy was defined as effects induced by the H + N combination that were statistically greater than the sum of the effects of the two separate treatments alone. To eliminate marginal but statistically significant synergies, a further more stringent requirement was imposed, namely that the H + N effect be equal to or greater than 1.3-times the calculated sum of the individual H and N effects. Since gene expression can be either up- or down-regulated by treatment, synergies can occur in either direction; therefore, absolute values were used to take into account consistent directionality. The statistical filtering criteria shown below in Table 1 were used to determine up- and down-regulation.

Table 1. Filtering applied to each probe set in the microarray to identify statistically significant synergies between H and N resulting in enhanced up-regulation or down-regulation of gene expression

Filtering criteria	Description
ANOVA H+N interaction $p < 0.05$	*Identifies significant interactions between H and N, requiring significance of synergism at $p < 0.05$*
$\text{Log}_2 \lvert(HN/VC)\rvert > 0$	*Fold-change calculation to screen for up- or down regulation by the H+N combination (VC = vehicle control)*
$\lvert HN - VC\rvert \geq 1.3^*\lvert(N - VC) + (HP - VC)\rvert$	*Filter for the stringent requirement that effects be 30% greater than the calculated additive levels for H and N*

An approach called 'theme analysis' was utilized to identify biological patterns in the lists of genes resulting from the synergy filtering. Theme analysis uses gene

functional annotation from the Gene Ontology (GO) Consortium[11]. This international group has developed an ontology of controlled vocabulary terms that describe the biological processes, molecular functions and cellular components associated with specific genes and their associated mRNAs. Theme analysis involves statistical comparison of a regulated list of genes and a larger reference list of all the expressed genes in an experiment, to determine if genes annotated to specific GO terms are significantly enriched in the regulated list. This type of analysis reveals biological patterns when multiple genes involved in a given process occur on the regulated list at a frequency greater than expected by chance. Theme analysis was performed using proprietary software called the Theme Extractor. This approach and statistical methods are similar to Gene Set Enrichment Analysis, which has been described in the literature[12].

6. Details of Data and Statistical Analysis

The table below shows the number of expressed mRNAs, indicated as the genes they are derived from, that met the stringent filtering criteria for statistically significant ($p < 0.05$) synergistic interactions between H and N in the epidermis of the skin equivalent cultures.

Table 2. Numbers of genes demonstrating a statistically significant synergy between Hexamidine and Niacinamide

Directionality	Number of genes
Up	106
Down	236

11 Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000 25:25-29.

12 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U.S.A* 2005 102:15545-15550.

There were more than 300 genes in the epidermis for which H + N had synergistic effects on expression, including both up- and down-regulated genes. Generally, the synergistic effects detected were substantial. The majority of the genes in the table above had H + N effects that were more than 3-times greater than predicted by additivity. This level of change is unusual and unanticipated, since many consequential regulatory changes in cells that are ususually seen involve smaller changes in gene expression, in the 10's of percents; 3-fold changes are profound, and can be expected to have large cascading overt effects in improving the condition of keratinous tissues such as skin.

To identify biological patterns associated with the genes synergistically regulated by H + N, the lists of epidermal genes showing synergies were subjected to theme analysis as described above for biological processes. The results of the theme analysis are shown in Table 3 below.

Table 3. Biological Process Theme Analysis of genes synergistically regulated by Hexamidine + Nicinamide in the epidermis of skin equivalent cultures

	Genes annotated to GO Terms	
Gene Ontology (GO) Biological Process Terms	Down-regulated	Up-regulated
GO:0008150 biological_process	214	86
.... GO:0046165 alcohol biosynthetic process	0	4**
..... GO:0048364 monosaccharide biosynthetic process	0	4**
..... GO:0019319 hexose biosynthetic process	0	4**
..... GO:0006094 gluconeogenesis	0	4***
..... GO:0006090 pyruvate metabolic process	0	4**
..... GO:0044257 cellular protein catabolic process	12*	0
..... GO:0051603 proteolysis involved in cellular protein catabolic process	12*	0
..... GO:0019941 modification-dependent protein catabolic process	12*	0
..... GO:0006511 ubiquitin-dependent protein catabolic process	12*	0
..... GO:0043632 modification-dependent macromolecule catabolic process	12*	0
..... GO:0006464 protein modification process	53*	13
..... GO:0043687 post-translational protein modification	47*	13
..... GO:0016567 protein ubiquitination	12*	3
..... GO:0006512 ubiquitin cycle	25**	5
..... GO:0030163 protein catabolic process	15*	0
.... GO:0043412 biopolymer modification	53*	14
..... GO:0000398 nuclear mRNA splicing, via spliceosome	2	7**
..... GO:0008380 RNA splicing	5	8*
..... GO:0000375 RNA splicing, via transesterification reactions	2	7**
Term p values: * p≤0.001; ** p≤0.0001; ***p≤0.00001		

The output indicates GO biological process terms that were statistically significantly associated with the regulated gene lists. The level of indentation indicates the level in the GO hierarchy and derivative relationships between terms; Biological Process (GO:0008150) is the highest level term. The numbers in the gene list columns are the numbers of genes annotated to the specific GO terms in each row of the table. For the up- and down-regulated lists, 86 and 214 genes had GO annotation, respectively. In addition, there were other genes synergistically affected by the combination of H + N, as indicated by the totals in Table 2, however these have not yet been associated with GO terms[11], and so are not included in Table 3. The p values for the GO term and gene list combinations are indicated by the asterisks in Table 3.

It appeared that genes synergistically affected by the combination of H + N are not random in biological process, but are clustered in specific processes. H + N unexpectedly caused a synergistic up-regulation of the expression of genes involved in two major biological processes. One of the major biological processes is gluconeogenesis [GO:0006094], the process of generation of glucose from non-sugar carbon substrates like pyruvate, lactate, glycerol, and glucogenic amino acids[13]. Glucose is a critical compound in cells, as both a source of energy and a metabolic intermediate. Glucose is a fundamental compound that is essential to processes that regulate the condition of mammalian keratinous tissues such as skin. These processes would be expressed overtly in preventing, retarding, and/or treating the appearance of skin conditions such as: fine lines and/or wrinkles; hyperpigmentation such as post-inflammatory hyperpigmentation; sagging; skin atrophy; skin dryness; dark under-eye circles and puffy eyes; sallowness; desquamating, exfoliating, and/or increasing skin turnover; enlarged pores; and/or oily and/or shiny appearance of skin.

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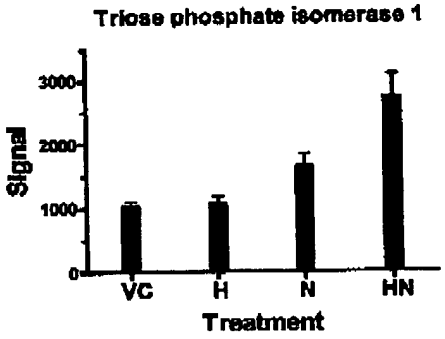
13 Lehninger AL, Nelson DL, Cox MM. *Principles of Biochemistry*, 4th Edition. W H Freeman & Co, Boston MA. 2004.

Below is an example of a gene involved in gluconeogenesis, triose phosphate isomerase, whose expression was synergistically up-regulated by the combination of H + N. Hexamidine by itself did not affect gene expression, and Niacinamide produced a 70% increase as compared to the vehicle control (VC). In combination, H + N increased triose phosphate isomerase gene expression to 260% of control. This combined effect indicated a significant interaction ($p = 0.047$, with significance set at $p < 0.05$) by ANOVA analysis, and a significant increase as compared to Niacinamide ($p = 0.028$) alone:



ANOVA HN interaction p = 0.047
|HN – VC| + |(N – VC) + (H – VC)| = 2.6

t-test p values:
HN vs VC p = 0.000064
HN vs N p = 0.028
N vs VC p = 0.032

H + N also synergistically up-regulated genes associated with a second major biological process, namely RNA splicing [GO:0008380]. RNA splicing is a critical enzymatic processing of RNA after it is transcribed from DNA, producing mature mRNA that undergoes translation as part of protein synthesis to produce proteins[13]. In addition, the combination of H + N also acted synergistically to significantly down-regulate a large number of genes involved in protein catabolic processes [GO:0044257], including genes involved in protein ubiquitination [GO:0006512]. Ubiquitination is the modification of proteins by covalent binding of the compound ubiquitin, which leads to degradation of the proteins by proteosomes as well as altered

stability, function and localization of a wide range of proteins[14]. This effect would lead to an unanticipated stabilization of cellular proteins. Processes that increase mRNA splicing and decrease protein catabolism are fundamental to processes that regulate the condition of mammalian keratinous tissues such as skin, and provide skin structural protein changes essential toward preventing, retarding, and/or treating overt changes such as the appearance of fine lines and/or wrinkles; sagging; skin atrophy; skin dryness; desquamating, exfoliating, and/or increasing skin turnover; and regulating and/or reducing the size of pores.

The theme analysis indicates that the genes unexpectedly regulated in a synergistic manner by the combination of H + N are not random from a biological function standpoint, since a large number of these genes are enriched in their association with the processes of gluconeogenesis; RNA splicing; and protein catabolism and the ubiquitin cycle.

7. Conclusions

Impressively, the results of the experiment described in this declaration indicate unexpected synergistic actions of the combination of Hexamidine + Niacinamide on genes expressed in an ***in vitro*** human skin epidermal model:

• The combination of Hexamidine + Niacinamide significantly ($p < 0.05$, ANOVA statistical analysis) synergistically increased or decreased expression of over 300 genes, as determined by stringent criteria including that the level of gene expression in response to the Hexamidine + Niacinamide combination was equal to or greater than 1.3-times the calculated sum of the individual H and N effects.

• The magnitude of the synergistic effects of Hexamidine + Niacinamide was substantial, with the majority of the affected genes being expressed at 3-times greater than predicted by additivity. This level of change is unusual and unanticipated, since

[14] Hershko A. Ubiquitin: roles in protein modification and breakdown. *Cell* 1983 Aug 34(1):11-12.

many consequential regulatory changes in cells that are usually seen involve smaller changes in gene expression, in the 10's of percents; 3-fold changes are profound, and can be expected to have large cascading overt effects in improving the condition of keratinous tissues such as skin.

• The genes affected by the combination of Hexamidine + Niacinamide were not random in terms of biological function, since unexpectedly a large number of these genes were statistically significantly ($p < 0.001$ or less) enriched in their association with the processes of gluconeogenesis; RNA splicing; and protein catabolism and the ubiquitin cycle, as determined by gene ontology (GO) analysis of the genes significantly affected by the combination of Hexamidine + Niacinamide as compared to either compound alone. Thus, the combination of Hexamidine + Niacinamide affected gene pathways involved in *increased glucose for energy metabolism*, *increased processing of RNA to enable protein synthesis*, and *stabilization of proteins*.

Increased glucose for energy metabolism is fundamental to processes for regulating the condition of mammalian keratinous tissues such as skin. These processes would be expressed overtly in preventing, retarding, and/or treating skin conditions including the appearance of fine lines and/or wrinkles; hyperpigmentation such as post-inflammatory hyperpigmentation; sagging; skin atrophy; skin dryness; dark under-eye circles and puffy eyes; sallowness; desquamating, exfoliating, and/or increasing skin turnover; enlarged pores; and/or oily and/or shiny appearance of skin.

Increased processing of RNA to enable protein synthesis and *stabilization of proteins* are fundamental to processes that regulate the condition of mammalian keratinous tissues such as skin, and provide skin structural protein changes essential toward preventing, retarding, and/or treating skin conditions such as the appearance of fine lines and/or wrinkles; sagging; skin atrophy; skin dryness; desquamating, exfoliating, and/or increasing skin turnover; and regulating and/or reducing the size of pores.

Appl. No. 10/780,267
Atty. Docket No. 9176R
Customer No. 27752

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true. This declaration is made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and may jeopardize the validity of the above-captioned patent application or any patent issuing thereon.

By: Rosemarie Osborne March 31, 2008
[Declarant] Date

18 U.S.C. §1001 - Whoever, in any matter within the jurisdiction of any department or agency of the United States knowingly and willfully falsifies, conceals or covers up by any trick, scheme, or advice a material fact, or makes any false, fictitious or fraudulent statement or representation, or makes or uses any false writing or document knowing the same to contain any false, fictitious or fraudulent statement or entry, shall be fined not more than $10,000 or imprisoned not more than five years, or both.